Oceanibacterium hippocampi gen. nov., sp. nov., isolated from cutaneous mucus of wild seahorses (*Hippocampus guttulatus*)

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Running title: *Oceanibacterium hippocampi* gen. nov., sp. nov.

Abstract

A Gram-negative, aerobic, motile and slightly curved rod-shaped bacterium (BFLP-8^T) was isolated from cutaneous mucus of wild long-snouted seahorses (*Hippocampus guttulatus*) captured in northwest Spain (Toralla, Galicia). Strain BFLP-8^T grew at 10–35°C and pH 5–9 (optimally at 25°C and pH 7.0) and with 1–6% (w/v) NaCl (optimally with 2% NaCl). The predominant respiratory quinone (90%) was ubiquinone with ten isoprene units (Q-10) and the major fatty acids identified were $C_{18:1}\omega7c$ (54.8% of the total), $C_{19:0}$ cyclo $\omega8c$ (11.6%), $C_{16:0}$ (9.5%), $C_{18:1}$ 2-OH (7.1%) and $C_{16:1}\omega11c$ (6.7%). The G+C content of the DNA was 57.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain BFLP-8^T formed a distinct clade within the family *Sneathiellaceae* but is not specifically associated with any species in the family. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain BFLP-8^T represents a novel species within a new genus, for which the name *Oceanibacterium hippocampi* gen. nov., sp. nov. is proposed. The type strain is BFLP-8^T (= CECT 7691^T = DSM 23444^T).

Keywords: *Oceanibacterium hippocampi*; polyphasic taxonomic analysis; seahorses

Introduction

The class *Alphaproteobacteria* comprises a large group of Gram-negative bacteria within the phylum *Proteobacteria* and is currently divided into ten orders: *Caulobacterales*, *Kiloniellales*, *Kordiimonadales*, *Parvularculales*, *Rhizobiales*, *Rhodobacterales*, *Rhodospirillales*, *Rickettsiales*, *Sneathiellales* and *Sphingomonadales* (www.bacterio.cict.fr). These bacteria show a wide range of morphological, physiological and genetic characteristics (Batut *et al*. 2004).

The family *Sneathiellaceae* in the order *Sneathiellales* was proposed by Kurahashi *et al*. (2008) based on phylogenetic analysis of 16S rRNA gene sequences. This family is currently represented by one genus with two species: *Sneathiella chinensis*, isolated from coastal sediments in China (Jordan *et al*. 2007) and *Sneathiella glossodoripedis*, isolated from the foot epidermis of a nudibranch in Japan (Kurahashi *et al*. 2008). In the present study, we describe the physiological, chemotaxonomic and phylogenetic characteristics of a novel strain, BFLP-8^T, belonging to a new genus of the family *Sneathiellaceae*.

Materials and Methods

Strain isolation

During the characterization of organisms isolated from cutaneous mucus of wild long-snouted seahorses (*Hippocampus guttulatus*), strain BFLP-8^T was isolated on marine agar (Difco, Becton Dickinson, Heidelberg, Germany) at 20ºC. The organism was subcultured on the same medium at 25ºC for 3 days. Stock cultures were stored at –80 ºC in marine broth (Difco) with 30% (v/v) glycerol.

Phenotypic tests

Gram reaction was determined using the non-staining (KOH) method as described by Buck (1982). Cell morphology and motility were studied using phase-contrast

77 microscopy and electron microscopy as previously described by Herrera *et al*. (2007).
78 NaCl growth tolerance and requirements were investigated by using nutrient broth
79 [0.5% peptone from casein, 0.3% meat extract, 0.3% yeast extract, adjusted to pH 7.2]
80 supplemented with various concentrations of NaCl (0–15% at intervals of 1%). The pH
81 range for growth was determined in marine broth that was adjusted to various pH values
82 with acetic acid-sodium acetate (pH 4.0-4.5, 100 mM), MES (pH 5.0-6.0, 50 mM),
83 MOPS (pH 6.5, 50 mM), Tris (pH 7.0-9.0, 50 mM) or CHES (pH 9.5-10.0, 50 mM)
84 buffers. Anaerobic growth was assessed at 25 ºC in anaerobic chambers with an H_2/CO_2
85 atmosphere (bioMérieux).
86 Catalase activity was determined by assessing bubble production in 3% (v/v) H_2O_2;
87 oxidase activity was determined using 1% (w/v) tetramethyl-*p*-phenylenediamine as
88 described by Lim *et al*. (2008). Some physiological characteristics were performed
89 using API 20NE and API ZYM (bioMérieux). Cells for inoculation of the strips were
90 grown for 3 days at 25ºC on marine agar and the results were visually interpreted
91 according to the manufacturer's instructions.
92 For base composition analysis, DNA was prepared according to Chun & Goodfellow
93 (1995) and the G+C content was determined using the thermal denaturation method
94 (Mandel & Marmur, 1968). DNA from *Escherichia coli* ATCC 11775^T was used as the
95 reference for determination of the thermal-melting profile (T_m).
96 **Chemotaxonomic analyses**
97 Whole-cell fatty acids from the isolate were extracted from biomass grown on marine
98 agar and were analysed according to the standard protocol of the Sherlock Microbial
99 Identification System (MIDI version 4.5). Analysis of respiratory quinones was carried
100 out by the DSMZ Identification Service and Dr. Brian Tindall (DSMZ, Braunschweig,
101 Germany).

Phylogenetic analysis

Extraction and amplification of genomic DNA for 16S rRNA sequence analysis were carried out as described previously (Balcázar *et al*. 2010). The sequences obtained were compared against the sequences available in the GenBank, EMBL and DDBJ databases obtained from the National Center for Biotechnology Information using the BLASTN (Altschul *et al*. 1990). Phylogenetic analysis was performed using the software MEGA version 5.0 (Tamura *et al*. 2011) after multiple alignments of data by CLUSTAL W (Larkin *et al*. 2007). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining and maximum-parsimony methods were determined using bootstrap values based on 1000 replications.

Results and Discussion

Cells of strain BFLP-8^T were slightly curved rods (Fig. 1), Gram-negative, oxidase- and catalase-positive, motile and aerobic. In addition, strain BFLP-8^T could be differentiated from its closest phylogenetic neighbours (see below) on the basis of some biochemical properties such as nitrate reduction, arginine dihydrolase and urease activities. Other physiological characteristics of strain BFLP-8^T are shown in Table 1 and also in the species description.

The DNA G+C content was calculated to be 57.8 mol%. This value corresponds closely to the range (56.9–57.1 mol%) reported for recognized members of the family *Sneathiellaceae* (Jordan *et al*. 2007; Kurahashi *et al*. 2008).

The predominant respiratory quinone of strain BFLP-8^T was Q-10 (90%), with Q-9 present in minor amounts (10%). Q-10 has been reported as the predominant quinone in many members of the *Alphaproteobacteria* (Wagner-Döbler *et al*. 2004), including species of the genus *Sneathiella* (Kurahashi *et al*. 2008). In addition, the fatty acid profile was composed of $C_{18:1}\omega7c$ (54.8%), $C_{19:0}$ cyclo $\omega8c$ (11.6%), $C_{16:0}$ (9.5%), $C_{18:1}$

127 2-OH (7.1%), $C_{16:1}\omega 11c$ (6.7%), 11-methyl-$C_{18:1}$ $\omega 7c$ (2.6%), $C_{18:1}$ $\omega 9c$ (2.2%), $C_{18:0}$
128 (2.0%) and $C_{18:0}$ 3-OH (2.0%). Fatty acids $C_{16:0}$ and $C_{18:1}\omega 7c$ are typically the major
129 fatty acids found in members of the family *Sneathiellaceae* (Kurahashi *et al*. 2008).
130 The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of
131 strain BFLP-8^T is FN687912. 16S rRNA gene sequence similarity calculations after a
132 neighbour-joining analysis indicated that strain BFLP-8^T grouped most closely with
133 uncultured bacterial clones (GenBank accession numbers FJ202709 and FJ205319, 92.6
134 and 92.4% sequence identity). The closest cultured relatives of strain BFLP-8^T were
135 *Oceanibaculum pacificum* MC2UP-L3^T (91.5%), *Sneathiella glossodoripedis* IAM
136 15419^T (91.1%), *Sneathiella chinensis* LMG 23452^T (90.9%) and *Oceanibaculum*
137 *indicum* P24^T (90.5%). Similar results were obtained with the maximum-parsimony
138 algorithm (Fig. S1). The phylogenetic tree suggested that the novel strain forms a
139 distinct clade within the family *Sneathiellaceae* but is not specifically associated with
140 any species in the family (Fig. 2). Phylogenetic analyses and the low levels of 16S
141 rRNA gene sequence similarities between the novel strain and other members of the
142 family *Sneathiellaceae* indicate that strain BFLP-8^T represent a new genus in the family.
143 Therefore, the phenotypic and genotypic properties of strain BFLP-8^T support its
144 description as a novel species within a new genus, for which the name *Oceanibacterium*
145 *hippocampi* gen. nov., sp. nov. is proposed.

146 **Description of *Oceanibacterium* gen. nov.**

147 *Oceanibacterium* (L. n. *oceanus*, the ocean; L. neut. n. *bacterium*, rod; N.L. neut. n.
148 *Oceanibacterium*, rod-shaped bacterium from the ocean [sea water]).
149 Gram-negative, motile, aerobic, non-spore-forming and rod-shaped. Cells grow at
150 temperatures in the range 10–35 °C. NaCl is required for growth. Positive for oxidase
151 and catalase activities. Nitrate is reduced to nitrite. The ubiquinone system is Q-10 and

the major fatty acids (>6%) are $C_{18:1}\omega7c$, $C_{19:0}$ cyclo $\omega8c$, $C_{16:0}$, $C_{18:1}$ 2-OH and $C_{16:1}\omega11c$. The type species is *Oceanibacterium hippocampi*.

Description of *Oceanibacterium hippocampi* sp. nov.

Oceanibacterium hippocampi (hip.po.cam'pi. L. gen. n. *hippocampi*, of the seahorse, isolated from *Hippocampus guttulatus*).

Displays the following properties in addition to those given in the genus description. Cells are slightly curved rod-shaped (0.4 × 1.0–1.6 μm). Optimum growth temperature is 25°C. No growth occurs below 10°C or above 35°C. Growth occurs at pH 5.0–9.0, but not below pH 4.5 or above pH 9.5. Growth occurs at NaCl concentrations between 1.0 and 6.0% (w/v) but not without NaCl or in the presence of >7.0% NaCl (w/v). Positive for arginine dihydrolase, urease and assimilation of adipate and malate. Negative for indole production, glucose fermentation, aesculin, gelatine hydrolysis, *β*-galactosidase and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, D-maltose, potassium gluconate, caprate, citrate and phenyl-acetate. API ZYM tests show activities for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase but not for trypsin, *α*-chymotrypsin, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *β*-glucosidase, *α*-mannosidase and *α*-fucosidase. The major fatty acids are $C_{16:0}$, $C_{16:1}\omega11c$, $C_{18:1}\omega7c$, $C_{19:0}$ cyclo $\omega8c$ and $C_{18:1}$ 2-OH; smaller amounts of $C_{18:0}$, $C_{18:1}\omega9c$, 11-methyl-$C_{18:1}\omega7c$ and $C_{18:0}$ 3-OH are present. The G+C content of the type strain is 57.8 mol% (T_m).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BFLP-8^T is FN687912.

The type strain, BFLP-8^T (= CECT 7691^T = DSM 23444^T), was isolated from cutaneous mucus of wild long-snouted seahorses (*Hippocampus guttulatus*).

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Table 1. Differential characteristics of strain BFLP-8^T and some related species

Characteristic	**1**	**2**	**3**	**4**	**5**
Oxidase	+	+	+	+	–
Growth without NaCl	–	–	+	+	+
Growth at 45 °C	–	+	–	+	–
Nitrate reduction	+	+	+	–	+
Arginine dihydrolase	+	+	–	+	–
Urease	+	–	+	+	–
Citrate utilization	–	+	–	–	–

Strains: 1, *Oceanibacterium hippocampi* gen. nov., sp. nov. BFLP-8^T; 2, *Sneathiella glossodoripedis* MKT133^T; 3, *Sneathiella chinensis* LMG 23452^T; 4, *Oceanibaculum pacificum* LMC2up-L3^T; 5, *Oceanibaculum indicum* P24^T. +, positive; –, negative. Data for 2-5 taken from Kurahashi *et al*. (2008), Jordan *et al*. (2007), Dong *et al*. (2010) and Lai *et al*. (2009), respectively.

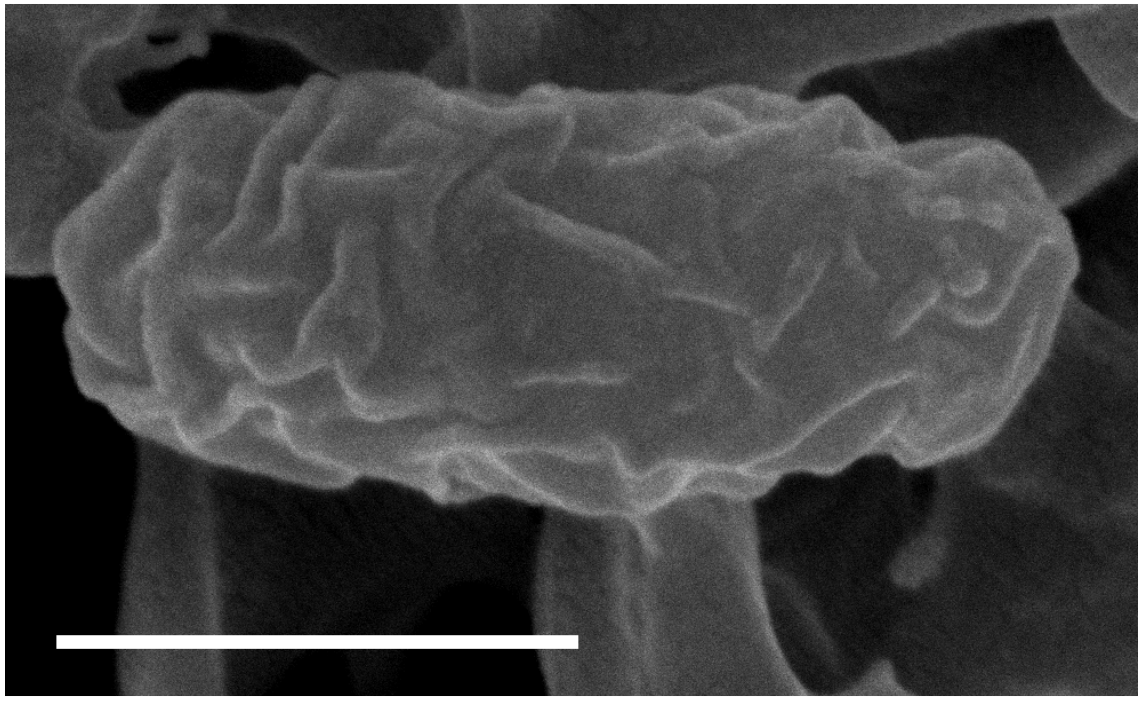


Fig. 1. Scanning electron micrograph of strain BFLP-8^T showing a slightly curved, rod-shaped morphology (0.4 × 1.0–1.6 µm). Bar, 0.5 µm.

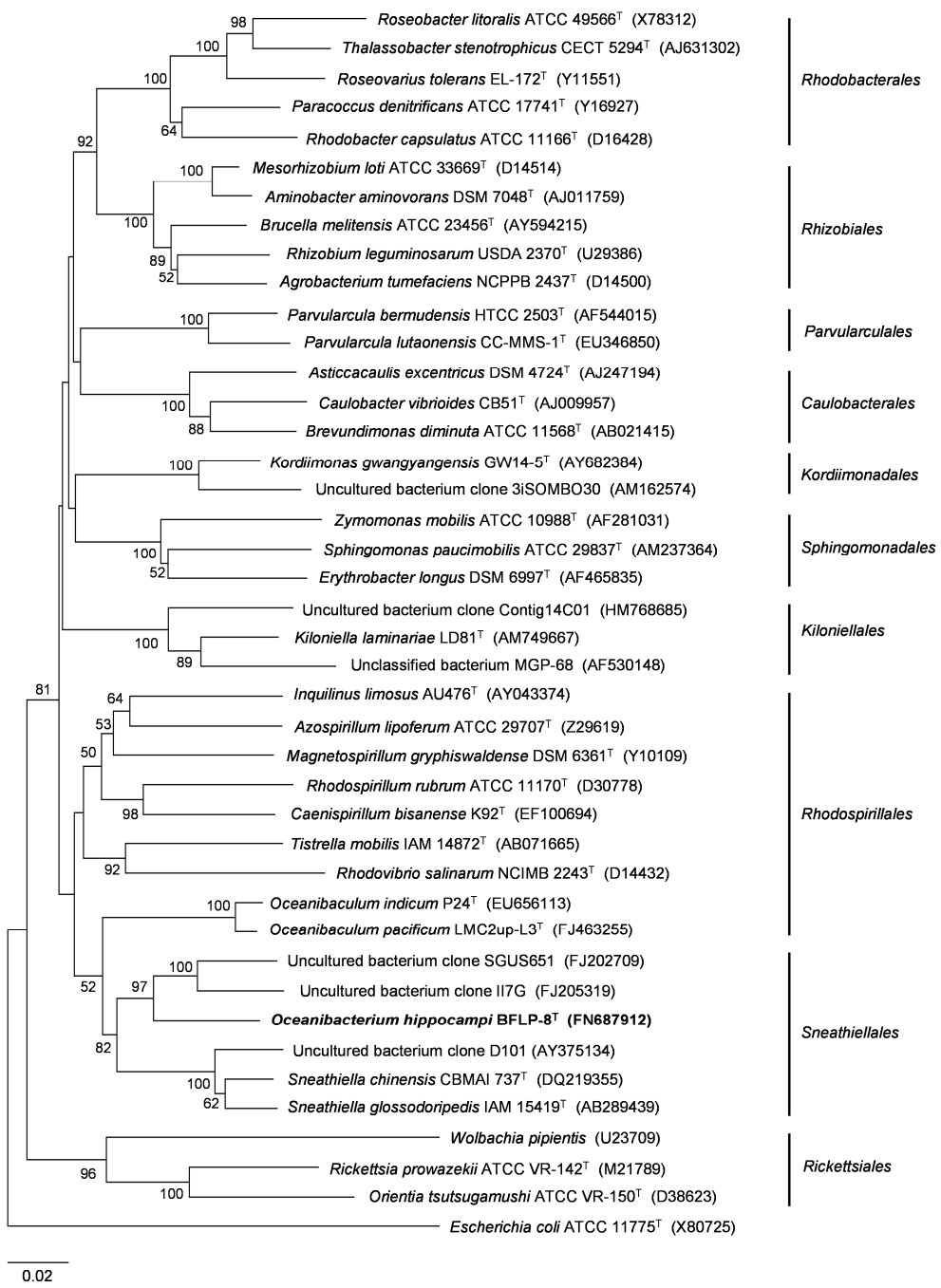
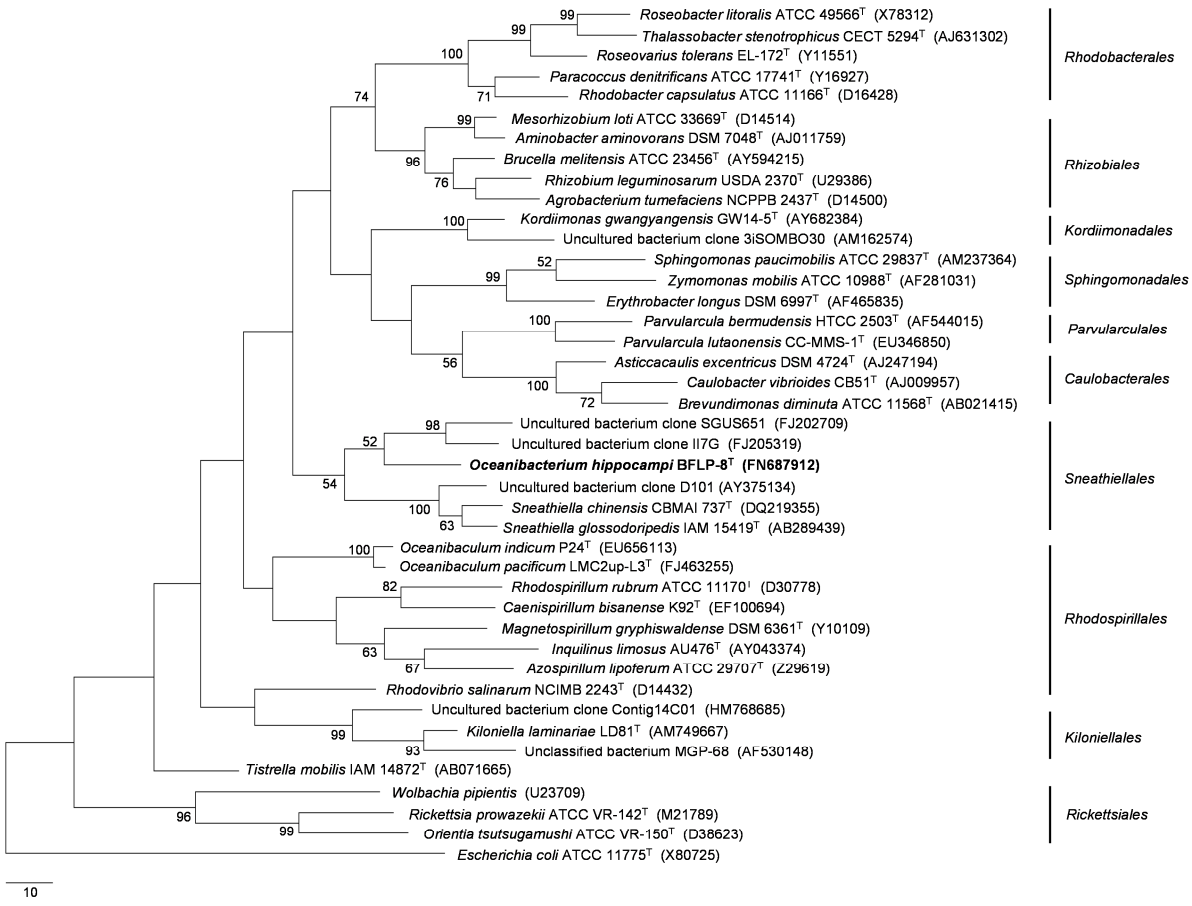


Fig. 2. Phylogenetic analysis of strain BFLP-8^T and representatives of the *Alphaproteobacteria* based on 16S rRNA gene sequences (GenBank/EMBL/DDBJ accession numbers in parentheses), constructed by the neighbour-joining method. Bootstrap percentages (>50%) based on 1000 replications are shown at branch nodes. *Escherichia coli* ATCC 11775^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.



Supplementary Fig. S1. Phylogenetic analysis of strain BFLP-8^T and representatives of the *Alphaproteobacteria* based on 16S rRNA gene sequences (GenBank/EMBL/DDBJ accession numbers in parentheses), constructed after multiple alignment of data by CLUSTAL W (Larkin *et al*. 2007). Distances were calculated and clustering with the maximum-parsimony method was performed by using the software package MEGA version 5.0 (Tamura *et al*. 2011). Bootstrap values based on 1000 replications are listed as percentages at branching points. *Escherichia coli* ATCC 11775^T was used as an outgroup. Bar, 10 substitutions per nucleotide position.

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